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STUDIES OF SUBSTRUCTURE AND TIGHTLY BOUND NUCLEOTIDE IN BACTERIAL MEMBRANE ATPase

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Highly purified preparations of Streptococcus faecalis ATPase contain a similar but inactive protein detected by prolonged polyacrylamide gel electrophoresis. The inactive protein appears to arise by proteolytic cleavage of the major subunits in the enzyme. By use of a new technique, subunit analysis in SDS gels was performed on the enzyme band and the inactive protein band excised from a polyacrylamide gel after electrophoresis. The results indicated that the ATPase has the composition $\alpha_3\beta_3\gamma$ in which α = 60,000, β = 55,000, and γ = 37,000 daltons. The inactive protein appears to have the composition $(f)_6$ in which f = 49,000 daltons. There is also evidence that the enzyme band contains some slightly modified forms of the ATPase, such as $\alpha_3\beta_2$ (f)γ. The inactive protein lacks the capacity for tight nucleotide binding.

Our experiments show that the tight ATPase-nucleotide complex formed in S. faecalis cells (the endogenous complex) behaves differently from the tight complex formed in vitro (the exogenous complex). We prepared a doubly labeled complex containing endogenous ^{32}P-labeled ADP and ATP and exogenous ^{3}H-labeled ADP. We observed that the addition of free nucelotide to the doubly labeled ATPase displaced the exogenous bound ligand from the enzyme but not the endogenous bound nucleotide. We suggest that the displaceable and nondisplaceable forms of the tight ATPase-nucleotide complex correspond to two different conformational states of the enzyme.

INTRODUCTION

In bacteria, an ATPase localized in the plasma membrane plays a key role in various energy-transducing processes (1, 2). The enzyme in Escherichia coli has a dual function. First, it mediates the synthesis of ATP coupled to respiration (3, 4). Second, by acting in the hydrolic direction, the enzyme also permits the utilization of ATP accumulation of various to drive ATP-dependent transhydrogenation reactions and the accumulation of various solutes (5–8). In S. faecalis, which is a homolactic fermentative organism lacking a cytochrome system, the membrane ATPase has a more restricted role. This organism does not ordinarily carry out oxidative phosphorylation and the function of the ATPase is confined to the utilization of glycolytically generated ATP for the energized uptake of certain solutes such as K^+ ions and amino acids (9–11). In none of these energy-transducing functions of the membrane-bound ATPase is the molecular mechanism understood.

Studies of the structural and catalytic properties of bacterial membrane ATPase began with the S. faecalis enzyme (2, 12, 13) and were gradually extended in many laboratories to a large variety of organisms. There is little doubt that the enzyme is

universally distributed in bacteria and that the basic features of its structure are the same in all bacteria. Indeed, the structure of the membrane ATPases in bacteria, mitochondria, and chloroplasts is virtually identical (2, 14).

In this paper we wish to report some new findings in our laboratory concerning two aspects of the S. faecalis ATPase. In the first part of the paper we will describe our recent investigations of the subunit structure of the enzyme and an inactive form of the enzyme which we believe arises as a result of limited proteolysis.

The second part of the paper extends our recent reports that the S. faecalis ATPase forms a stable tight complex with ADP and ATP within the cell and also in vitro (15, 16). Our experiments with the S. faecalis ATPase indicate that the ATPase-nucleotide complex that originates in the cell, which we will call the endogenous complex, is in a different physical state from that of the complex formed in vitro, which we will refer to as the exogenous complex. Using a doubly labeled preparation of the ATPase-nucleotide complex, we find that the endogenous protein-bound nucleotide (labeled with ^{32}P) is not displaceable by the free nucleotide. However, the exogenous protein-bound nucleotide (labeled with ^{3}H) is readily displaceable by the free ligand.

METHODS

Preparation of Endogenous ^{32}P-Labeled ATPase

S. faecalis cells were harvested in the stationary phase of growth from 2,400 ml of growth medium containing 1% tryptone, 0.5% yeast extract, 1% glucose, 0.8% KCl, and 3 mM K_2HPO_4. To label the ATPase with ^{32}P (endogenous label) the cells were washed and incubated for 30 min at 38°C in 100 ml of a solution containing 20 mM Tris buffer pH 7.5, 10 mM KCl, 1% glucose, and 20 mCi of carrier-free $^{32}P_i$ (New England Nuclear) (15). The cells were converted to protoplasts which were then lysed by osmotic shock (12). The resulting membranes were subjected to a series of aqueous washes which resulted in the selective release of the membrane-bound ATPase (13, 16). The enzyme was precipitated by dialysis against 80% saturated $(NH_4)_2SO_4$ and redissolved in 3 ml of 20 mM Tris buffer, pH 7.5, and 10 mM $MgCl_2$, and dialyzed against the same solution for 30 min. All operations were carried out at 4°C. Unlabeled ATP, Pi, and pyrophosphate (0.1 mM) were added during the formation and lysis of protoplasts and during the $(NH_4)_2SO_4$ precipitation step to help remove radioactive contamination.

Polyacrylamide Gel Electrophoresis

Electrophoresis was done in vertical 5% polyacrylamide slabs with a Tris-glycine buffer at pH 8 (regular gel). To locate the band with ATPase activity, we employed a staining procedure described by Abrams and Baron (17) that makes use of the Fiske-SubbaRow reagents to detect Pi produced when the gel is soaked in MgATP.

We devised a method for superimposing the standard protein stain on a gel that had already been stained for ATPase activity. As soon as the blue-colored band appears indicating the position of the ATPase, the slab is immersed in a 1:1 benzene-isobutanol mixture until the blue color disappears and the gel turns yellow (about 30 min). The gel is then rinsed with water for a few minutes and stained for protein overnight with 0.05% Coomassie blue in CH_3OH, H_2O, and acetic acid (5:5:1). Destaining is accomplished

with the same solvent. An example of protein staining superimposed over ATPase staining is illustrated in Fig. 8. It is obviously very useful for distinguishing a catalytically active band from a nearby inactive protein.

Electrophoresis in sodium dodecyl sulfate (SDS) was carried out essentially as described by Weber and Osborn (18). We used a 10% polyacrylamide slab containing 10 mM sodium phosphate, pH 7, and 0.1% SDS. The same solvent was placed in the buffer compartments. The sample was dissolved in 5% SDS, 10% mercaptoethanol, and 10 mM phosphate buffer, pH 7, and placed in boiling water for a few minutes.

Subunit Analysis of Protein Bands Excised from Gels

The protein is electrophoresed in a 3 mm thick regular gel and stained with Coomassie blue in the usual fashion. The protein band is excised from the slab and the gel slice is dehydrated by immersing it in a 1:1 methanol-ethanol mixture for about 1 hr. It is then soaked overnight at 38°C in about 200 μl of 5% SDS, 10% mercaptoethanol, and 100 mM phosphate buffer, pH 7, to dissociate the protein into its subunits. The gel piece is then inserted with the aid of a syringe needle into the slot of a 6 mm thick SDS gel slab and covered with a layer of 10% sucrose dissolved in compartment buffer. After 1 hr the current is turned on. We have shown that electrophoresis of subunits from a gel piece gives the same mobilities as a sample of subunits in solution. This method is useful, for it permits subunit analysis of discrete protein bands which usually can be considered to be absolutely pure. If there is doubt about the purity of the protein band the protein can be purified once again through regular gels with the excision procedure. This was actually done in our analysis of ATPase subunits shown in Fig. 3.

Paper Electrophoretic Analysis of Labeled Nucleotides in the ATPase

Samples of the labeled ATPase, about 3 units in 1.0 ml, were dialyzed against 0.2 mM MgCl and acidified to about pH 3. About 200 μg pepsin was added and the mixture was incubated at 38°C for 5 hr. The pH was lowered to about 2 and centrifuged to remove insoluble material. The sample was reduced in volume by warming under a N_2 stream to about 40 μl and electrophoresed at pH 3.8 (16) on a 30-cm sheet of Whatman 3 MM paper for 5 hr at 620 V with a water-cooled apparatus. Unlabeled ATP, ADP, AMP, and Pi, about 5 μg each, were added to the sample as carrier prior to electrophoresis. Following electrophoresis the nucleotide spots were located by UV light and Pi was located by comparison to a standard mixture with $^{32}P_i$ run alongisde the sample. The paper was then sectioned and each piece was soaked in 0.7 ml H_2O in a scintillation vial. Scintillation fluid containing one part triton to three parts toluene was added and the content of ^{3}H and ^{32}P was determined.

Other Methods

Zonal sedimentation was carried out as in earlier work (16). The ATPase was assayed as previously described (13). One unit of enzyme is that amount which yields 1 μmol/min.

RESULTS

Active and Inactive Forms of the ATPase

Despite extensive purification by gel filtration, preparations of the S. faecalis ATPase

usually contain some catalytically inactive protein which is electrophoretically very similar to the enzyme in polyacrylamide gels. As illustrated in Fig. 1, the resolution of the two proteins requires prolonged electrophoresis. Tests of the enzyme activity directly on the gel, according to the method of Abrams and Baron (17), show that only the slower moving protein is catalytically active.

The fact that the ATPase and the inactive protein copurify on very long gel filtration columns and migrate closely during polyacrylamide gel electrophoresis indicates that they are very similar in size and charge. This led us to suspect that the inactive protein might be a product formed by proteolytic removal of a small piece or pieces of the ATPase polypeptide chains. We tested this possibility by treating a purified ATPase preparation with varying amounts of trypsin and examining the outcome by gel electrophoresis. The result illustrated in Fig. 2 shows that the enzyme is converted quantitatively to an inactive protein whose mobility is identical to that of the naturally occurring inactive protein (Figs. 1, 2). Only very low levels of trypsin were required for this conversion to the inactive protein. As can be seen in Fig. 2, the inactive protein product of trypsin action is quite resistant to further proteolytic degradation as very high levels of trypsin failed to cause any discernible change in its amount. Therefore, it seemed that the natural inactive protein could very well be a product of the action of cellular proteases on the ATPase. Phenylmethyl sulfonyl fluoride added during the preparative procedures failed to prevent the appearance of the inactive protein. This suggests that the ATPase might have been modified within the cell.

Subunit Composition of the ATPase and the Inactive Protein

Some time ago we reported that the S. faecalis ATPase has a molecular weight of 385,000 daltons (19). From polyacrylamide gel analyses of the purified enzyme in 6 M urea we concluded that the enzyme was a multimeric protein containing two major types of subunits called α and β, and possibly a minor subunit called γ (17, 19, 20). Measurements of the subunit size by high-speed sedimentation equilibrium of the enzyme in 6 M guanidinium chloride yielded a value of 33,000 daltons. However, we believe that this earlier estimate of the subunit size is probably incorrect, as further work using SDS-gel analysis as described in this paper yields considerably higher values (2) (Fig. 3).

Before a valid analysis of the subunit composition of the ATPase could be undertaken it was first necessary to separate the enzyme from the contaminiating inactive protein. At present this can be done only by polyacrylamide gel electrophoresis (Fig. 1). Instead of eluting the enzyme band from the gel, we developed a new technique whereby the subunits of the individual protein bands in the gel could be determined without prior removal of the protein from the gel. (For details see Methods.) It should be noted that the SDS gel analyses of the ATPase and the inactive protein were carried out side-by-side on the same slab so that their composition could be reliably compared. The results (Fig. 1) show that the ATPase band consisted of two major polypeptides designated α and β and two minor polypeptides designated f and γ. The gel-purified inactive protein, on the other hand, yielded only a single type of polypeptide which matched the minor component, f, in the ATPase (Fig. 1). The molecular weights of the α, β, f, and γ polypeptides in the ATPase were evaluated by comparison with a set of standards as shown in Fig. 3 following the procedure of Weber and Osborn (18). The results were as follows: α = 60,000; β = 55,000; f = 49,000; and γ = 37,000.

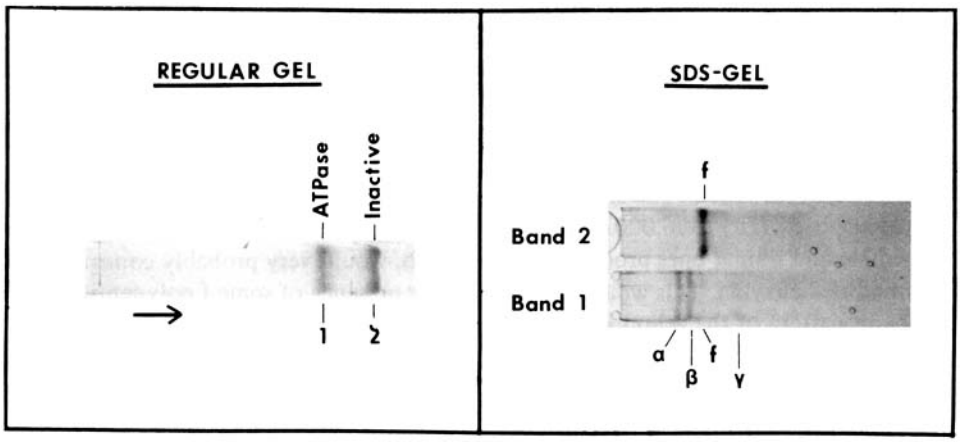

Fig. 1. Electrophoretic resolution of ATPase and inactive protein and determination of their respective subunits. The SDS gel analysis was done on the protein bands excised from the regular gel (see Methods).

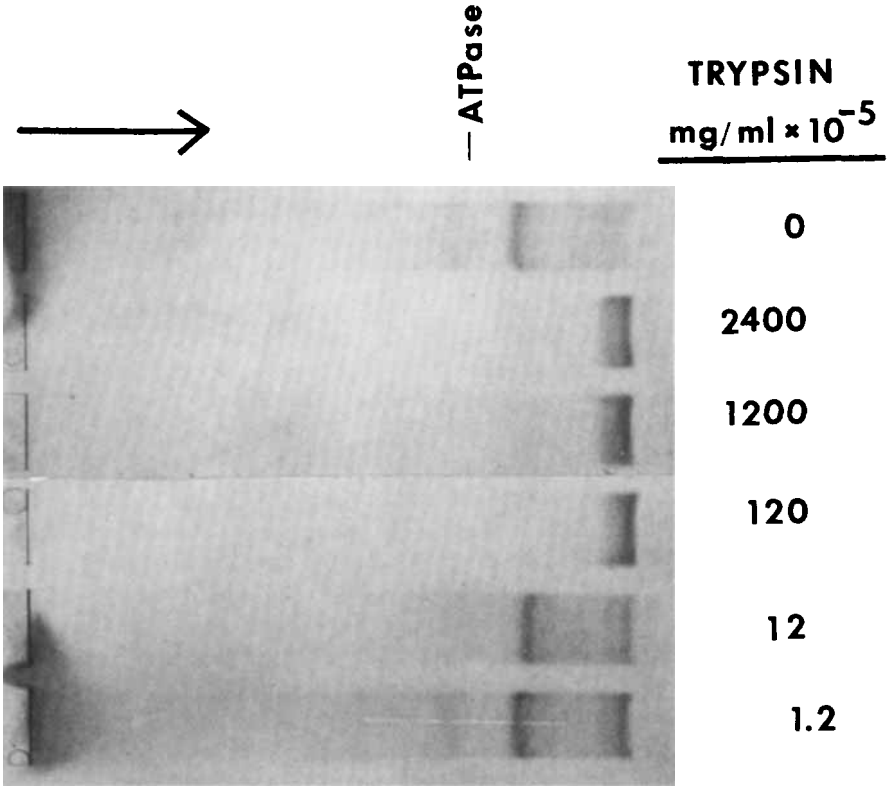

Fig. 2. Formation of inactive protein from the ATPase by trypsin. The ATPase preparation was treated with the levels of trypsin indicated for 1 hr at 38°C. The treated samples and control were then analyzed by electrophoresis in regular gels.

To account for these findings, particularly the presence of f in both the active enzyme and the inactive protein, we have formulated the scheme shown in Fig. 4. This scheme proposes that the enzyme has the subunit composition $\alpha_3\beta_3\gamma$ (mol wt 382,000 daltons) and that it is cleaved by a protease near the terminus of the α and β chains. The site of cleavage is such as to produce from α (60,000) and β (55,000) an identical or nearly identical fragment, f = 49,000 daltons, which remains associated with the enzyme. As illustrated in the scheme the cleavage of only one β chain in the ATPase would form the molecule $\alpha_3\beta_2$ (f) γ (376,000 daltons). Since this sort of modification is so slight, such an intermediate and similar ones produced by cleaving α, would very probably comigrate with the unmodified enzyme. This would account for the presence of some f polypeptide in the subunit pattern of the gel-purified ATPase band (Figs. 1 and 3). As shown in the scheme, eventually all of the α and β chains are cleaved to yield the trypsin-resistant inactive protein with the composition $(f)_6$ (294,000 daltons). As indicated in the scheme, the minor subunit, γ (37,000), is lost since the inactive protein contains only the f polypeptides (Fig. 1, SDS gel slot #2).

Finally, we should like to point out that the subunits in $\alpha_3\beta_3\gamma$ and in $(f)_6$ are apparently associated by noncovalent forces. We draw this conclusion because the subunit pattern in SDS gels is unchanged if mercaptoethanol is omitted from the standard treatment used to dissociate the protein into its subunits.

In Table I we compare the molecular weights of the S. faecalis ATPase and its subunits with the values obtained for many other bacterial ATPases. The similarities are quite striking, except that in certain preparations one or more of the minor subunits is absent.

Tight Nucleotide Binding: Evidence for Displaceable and Nondisplaceable Forms

Preliminary studies in our laboratory indicated that bound nucleotide, [^{32}P]-ADP and [^{32}P]-ATP, found in the endogenous ATPase-nucleotide complex isolated from S. faecalis is not displaced by adding the free ligand (15, 16). In contrast to this unexpected behavior our studies of the exogenous complex indicated that the tightly bound nucleotide is readily displaced from the enzyme by adding the free ligand. These observations suggested that the endogenous and the exogenous complexes were not equivalent and therefore two forms of tight ATPase-nucleotide complex exist.

In our earlier studies we found that both the endogenous and the exogenous complexes are quite stable and easily isolatable in the absence of any free ligand by column chromatography, zonal sedimentation, or gel electrophoresis (15, 16). The dissociation constant of the exogenous ATPase-ADP complex, determined by equilibrium dialysis, was 2×10^{-7} M at pH 7.5 in the presence of EDTA (21); the stoichiometry is uncertain but there are probably at least two nucleotide binding sites per mole of enzyme (16, 21). For the endogenous complex a very tentative estimate of the stoichiometry yielded a value of 5–10 P atoms per mole of enzyme (15). It is important to mention that the exogenous and endogenous complex were stable in EDTA. Therefore, it seems that free multivalent cations are not required for tight nucleotide binding.

The experiment we wish to describe here was designed to provide more definitive evidence that the endogenous and exogenous ATPase-nucleotide complexes (15, 16) represent two different forms of tight nucleotide binding, namely a nondisplaceable and a displaceable form. In essence, our experimental approach was to prepare a doubly labeled

TABLE I. Molecular Properties of Bacterial Membrane ATPases

Source	Mol Wt	Subunit mol wt (1,000s) Major α	Major β	Minor	Minor γ	Minor σ	Minor ε	Authors
S. faecalis	385,000	60	55	(49)	37	–	–	Schnebli and Abrams, 1970) Abrams, this paper (19, 23)
E. coli (NCR482)	365,000-390,000	57	52		31	21	12	Davies and Bragg, 1972 Bragg and Hou, 1972; Bragg et al., 1973 (24–26).
E. coli B	360,000	60	56		35	–	13	Hanson and Kennedy, 1973 (27)
E. coli K12 (λ)	296,000	58	52		31	20	12	Futai et al., 1974; Nelson
	240,000	58	52		31	–	12	et al., 1974 (28, 29)
M. lysodeikticus	352,000	62	60		present			Salton and Schor, 1972, 1974 (30, 31)
	345,000	62	60		absent			
B. megaterium KM	379,000	68	65		–	–	–	Mirsky and Barlow, 1973 (32)

TABLE II. Selective Displacement of Bound Exogenous ^{3}H-ADP from ^{32}P-Labeled ATPase

Treatment	Bound ^{3}H specific activity (cpm/unit)	Bound ^{3}H % remaining	Bound ^{32}P specific activity (cpm/unit	Bound ^{32}P % remaining
None	2,595	(100)	4,722	(100)
Dialysis only	1,917	74	5,450	115
Displacement mixture* (0.1 mM) plus dialysis	433	17	5,200	110
Displacement mixture* (1.0 mM) plus dialysis	283	11	4,650	98

*The displacement mixture is nonradioactive AMP, ADP, ATP, and Pi in Tris-EDTA buffer at pH 7.5.

ATPase-ADP complex using ^{32}P to label the endogenous form and ^{3}H to label the exogenous form. With an enzyme containing both endogenous and exogenous bound ADP it becomes possible to test whether or not the exogenous ADP is selectively displaced by adding unlabeled ligand. In this initial test of differential displaceability we chose to use ADP rather than ATP to label the complex with exogenous nucleotide because we had previously observed that exogenous bound ATP undergoes extensive conversion to ADP (16). In the near future we plan to carry out a similar experiment with ATP.

The preparation of the doubly labeled ATPase and its isolation by gel filtration column chromatography is described in Fig. 5. ^{3}H-ADP was used as the exogenous labeled nucleotide. As can be seen both ^{32}P and ^{3}H were associated with the ATPase peak eluted from an agarose 1.5 m column. The enzyme was concentrated by ultrafiltration and then tested for selective displacement of the ^{3}H-ADP in the following manner. A sample of the enzyme was mixed with 1.0 mM or 0.1 mM unlabeled ATP, ADP, AMP, and Pi in Tris-EDTA

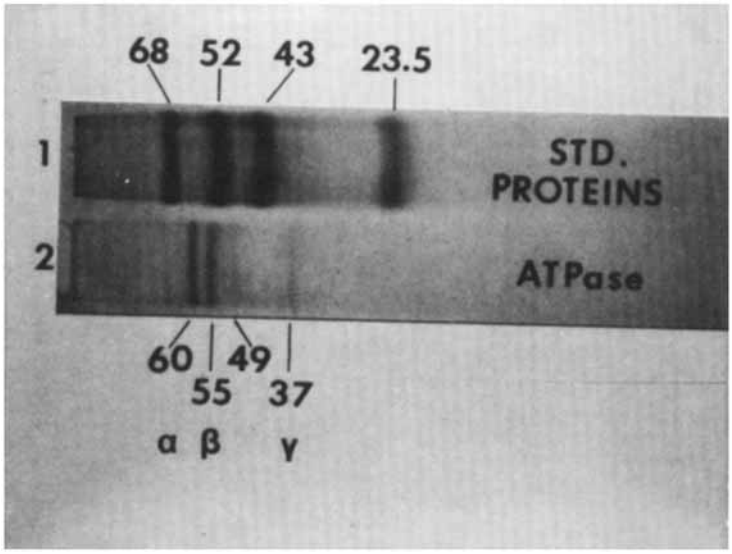

Fig. 3. Molecular weight determination of the S. faecalis ATPase subunits in SDS gels. The ATPase sample was purified twice in regular polyacrylamide gels by the "band excision" method (see Methods). The standard proteins were BSA (68,000), gamma globulin heavy chain (52,000), ovalbumin (43,000), and gamma globulin light chain (23,500).

buffer, pH 7.5, and then dialyzed. A control sample in Tris-EDTA buffer, to which no unlabeled nucleotide or Pi was added, was also dialyzed. The ^{32}P and ^{3}H content of the samples were measured before and after dialysis. The results are presented in Table II. They show clearly that the exogenous nucleotide ^{3}H-ADP was almost completely displaced by the addition of free nucleotide while there was no significant displacement of ^{32}P from the enzyme. The chemical nature of the labeled components in the treated and untreated sample after dialysis was then determined. The method used was paper electrophoresis after mild acidification of the enzyme to free the bound ligands (15). The results are depicted in Fig. 6. They show that the dialyzed untreated doubly labeled enzyme contained both [^{3}H]-ADP and [^{32}P]-ADP. On the other hand, the enzyme that was treated with unlabeled ATP, ADP, AMP, and Pi still retained the [^{32}P]-ADP but not the [^{3}H]-ADP. This result shows quite directly that the exogenous bound nucleotide, [^{3}H]-ADP, was selectively displaced from the doubly labeled enzyme by the free ligand. It is of interest also that the endogenous bound [^{32}P]-ATP was also not displaceable by the free ligand.

Confirmatory evidence for the selective displacement of exogenous bound ADP was obtained by zonal sedimentation analysis of the doubly labeled enzyme before and after treatment of the enzyme in Tris-EDTA buffer with a mixture of unlabeled ATP, ADP, AMP, and Pi (Fig. 7). As can be seen, before treatment with the unlabeled compounds, the enzyme peak contains both ^{3}H and ^{32}P. After treatment, the ^{3}H disappeared from the enzyme while the ^{32}P remained. As already shown (Fig. 6), the ^{32}P consists mainly of ^{32}P labeled ATP, ADP, and Pi.

Finally, we wish to point out that both the endogenous ^{32}P and the exogenous ^{3}H

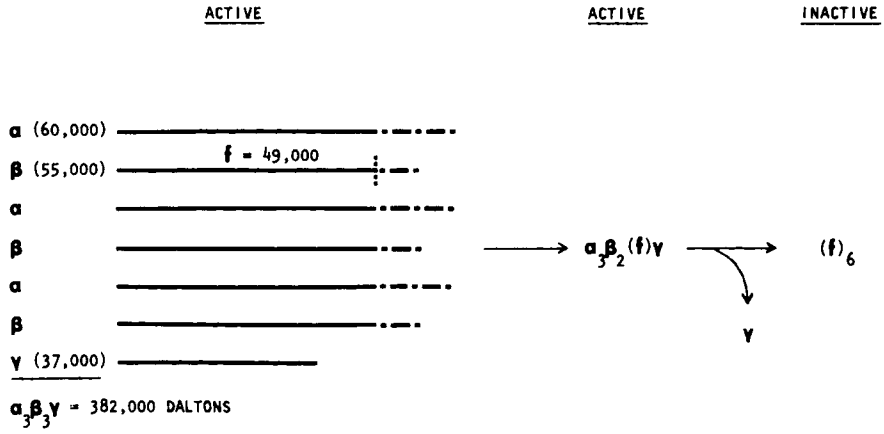

Fig. 4. Scheme of substructure and proteolytic scission of S. faecalis ATPase.

bound nucleotide in the doubly labeled ATPase are associated only with the active enzyme and not with the inactive protein. This is demonstrated by the radioactive profile of the electrophoretic pattern determined after prolonged gel electrophoresis of the doubly labeled enzyme in polyacrylamide gels as shown in Fig. 8.

DISCUSSION

Substructure of the ATPase

We have concluded that highly purified preparations of S. faecalis ATPase sometimes consist of the native enzyme mixed with some modified forms of the enzyme that arise by limited proteolytic cleavage of the major subunits (Figs. 1–4). We reached this conclusion mainly by examining the subunit composition of the active enzyme band and a faster moving inactive protein band that resolve when a purified ATPase preparation is subjected to prolonged electrophoresis in polyacrylamide gel. We were able to determine the subunit composition of each of these protein bands by SDS gel electrophoresis using a new technique which does not require prior removal of the protein from the gel (Figs. 1 and 3). The results of the subunit analysis indicate that the enzyme band is mostly the unmodified native ATPase ($\alpha_3\beta_3\gamma$) mixed with a small amount of slightly modified enzyme molecules such as $\alpha_3\beta_2$ (f) γ. The molecular weights of the polypeptide chains are α = 60,000, β = 55,000, f = 49,000, and γ = 37,000 daltons (Fig. 3).

The SDS gel analysis of the inactive protein band consisted of only f polypeptide chains (49,000 daltons) (Figs. 1 and 3). The available evidence (Figs. 1–3) suggests that the subunit composition of the inactive band is $(f)_6$ and that it is formed by limited proteolytic cleavage of all the α and β subunits in the native enzyme accompanied by the loss of the γ subunit. The change in molecular weight and charge in the transformation of

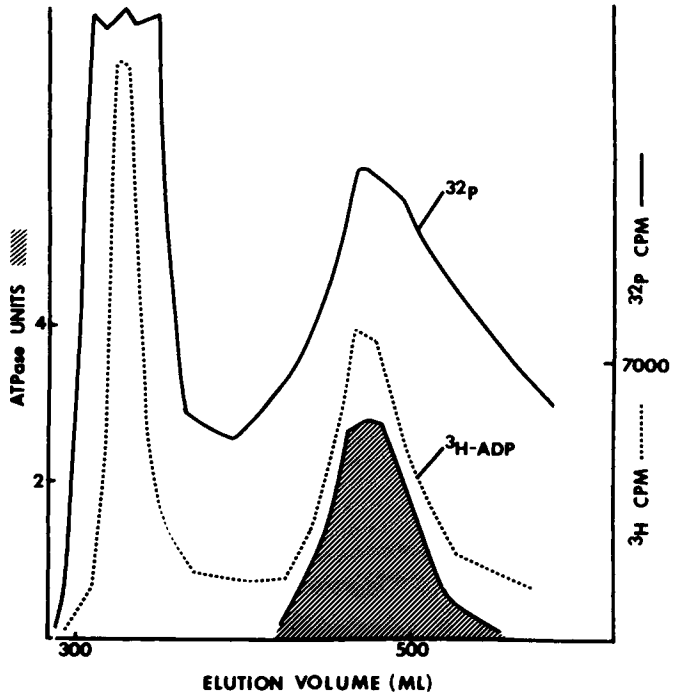

Fig. 5. Isolation of doubly labeled ATPase-nucleotide complex by gel filtration. About 40 units of ^{32}P-labeled ATPase (endogenous label; see Methods) were mixed with exogenous label 0.1 mM ^{3}H-ADP (6.7 × 10^{7} cmp/μmole) in a volume of 6 ml. The mixture also contained 0.1 mM unlabeled AMP and Pi and 40 mM EDTA. The pH was 7.5. After 1 hr at 4°C the mixture was chromatographed at 4°C on a 2.6 × 169 cm column of agarose (1.5 M) at a flow rate of 23 ml/hr using Tris-Mg buffer, pH 7.5, as the eluting fluid. Fractions (5.7 ml) were collected and assayed for ATPase activity and for ^{32}P and ^{3}H by liquid scintillation counting. About 25 ATPase units were recovered.

the native ATPase (385,000 daltons) to the inactive protein (294,000 daltons) is apparently sufficient to permit a clear resolution of the two by polyacrylamide gel electrophoresis.

A diagrammatic representation of our scheme of the transformation of the native enzyme to the inactive protein is given in Fig. 4. According to this proposal, limited proteolysis of the α and β chain yields the same or similar f polypeptides, i.e. 49,000 dalton polypeptides not resolvable in SDS gels (Fig. 1).

It is of interest to note that the molecular weight of the minor subunit γ is 37,000 daltons, essentially the same as that reported for nectin, a protein believed to be necessary for attachment of the ATPase to the membrane (20). We do not preclude the possibility that the γ polypeptide, like the f fragment, is a proteolytic cleavage product of the major subunits α and β.

Nucleotide Binding

Our studies of endogenous and exogenous bound nucleotide provide strong evidence that there are two types of tightly bound nucleotide in the S. faecalis ATPase. We use the

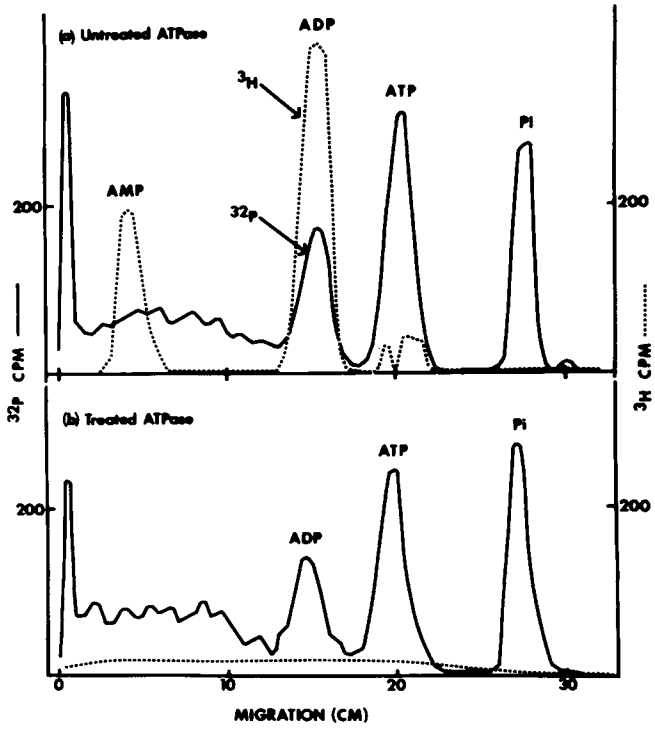

Fig. 6. Paper electrophoretic analysis of the labeled compounds in the ATPase. The labeled ATPase was acidified and analyzed as described under Methods. (a) Doubly labeled ATPase dialyzed but not treated with unlabeled compounds (from Table II). (b) Doubly labeled ATPase treated with 1 mM unlabeled ATP, ADP, AMP, and Pi and dialyzed (From Table II). Note that the exogenous label, ^{3}H-ADP, has been displaced from enzyme but not the endogenous label, ^{32}P-ADP.

terms "endogenous" and "exogenous" to distinguish between tightly bound nucleotide originating in the cell and tightly bound nucleotide that is formed in vitro. Experimentally, the endogenous bound nucleotide (^{32}P-ADP and ^{32}P-ATP) is not displaceable by the free nucleotide, while the exogenous bound nucleotide (^{3}H-ADP) is readily displaceable (Figs. 5–7) (Table II). Notably, the endogenous bound nucleotide survives the addition of free nucleotide at many steps during the isolation and purification of the enzyme. Using ^{3}H-ADP we can show that exogenous nucleotide binds to the isolated enzyme yet does not displace endogenous bound nucleotide (^{32}P-ADP) (Fig. 6). Moreover, the exogenous bound ^{3}H-ADP can in turn be displaced by adding unlabeled ADP, while the endogenous bound ^{32}P-ADP is not displaced (Fig. 6). It should be emphasized that an 0.1 mM concentration of free nucleotide was sufficient to displace exogenous bound ^{3}H-ADP. On the other hand, a level of free nucleotides as high as 1.0 mM would not displace the endogenous bound nucleotide (Fig..7). We must point out also that the concept of displaceable and nondisplaceable forms of tight nucleotide binding applies to both ADP and ATP and may apply to Pi as well (Figs. 6 and 7).

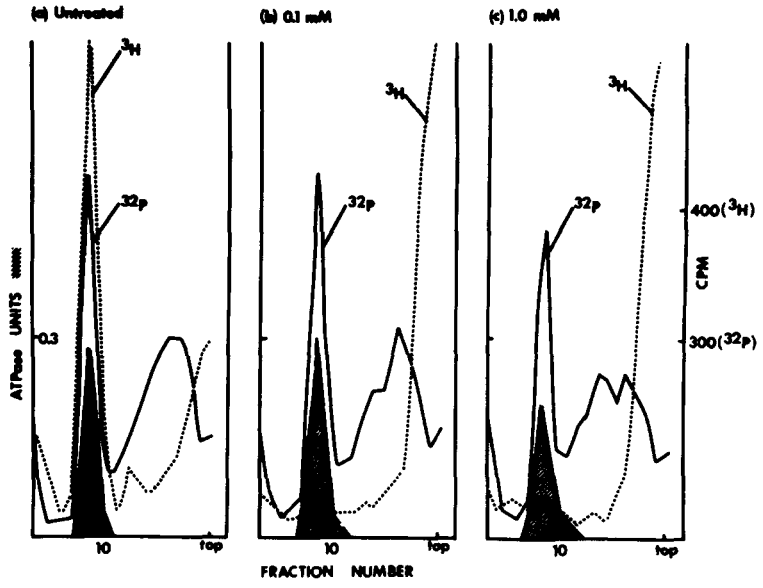

Fig. 7. Zonal sedimentation of the ATPase-nucleotide complex. (a) Doubly labeled ATPase with endogenous (^{32}P) and exogenous (^{3}H-ADP) label untreated. (b) Doubly labeled ATPase after treatment with 0.1 mM unlabeled ATP, ADP, AMP, and Pi. (c) Doubly labeled ATPase after treatment with 1.0 mM unlabeled ATP, ADP, AMP, and Pi.

At this time we can only speculate about the physical basis for nondisplaceable and displaceable forms of tight nucleotide binding that we have observed. We would like to propose that the nondisplaceable nucleotide behaves like an irreversibly bound ligand because it is sequestered in a pocket of a folded polypeptide chain which is not available to external exogenous nucleotide. This suggests that an alternative conformational state of the polypeptide chain exists in which the pocket is open, thus making the binding site available to exogenous nucleotide binding and displacement.

We would like to remark finally that the views presented here resemble certain postulates in the conformational coupling theory proposed by Boyer (22) to explain energy transduction in mitochondria. In the Cross and Boyer theory, as in the studies presented here, two conformational states of an energy-transducing ATPase corresponding to two types of nucleotide binding are thought to exist.

ADDENDUM

The membrane ATPase of Proteus L-forms has recently been characterized. It contains the major subunits α and β but no minor subunits. [Monteil, H., Roussel, G., and Boulois, D., Biochim. Acta, 382: 465–478 (1975)].

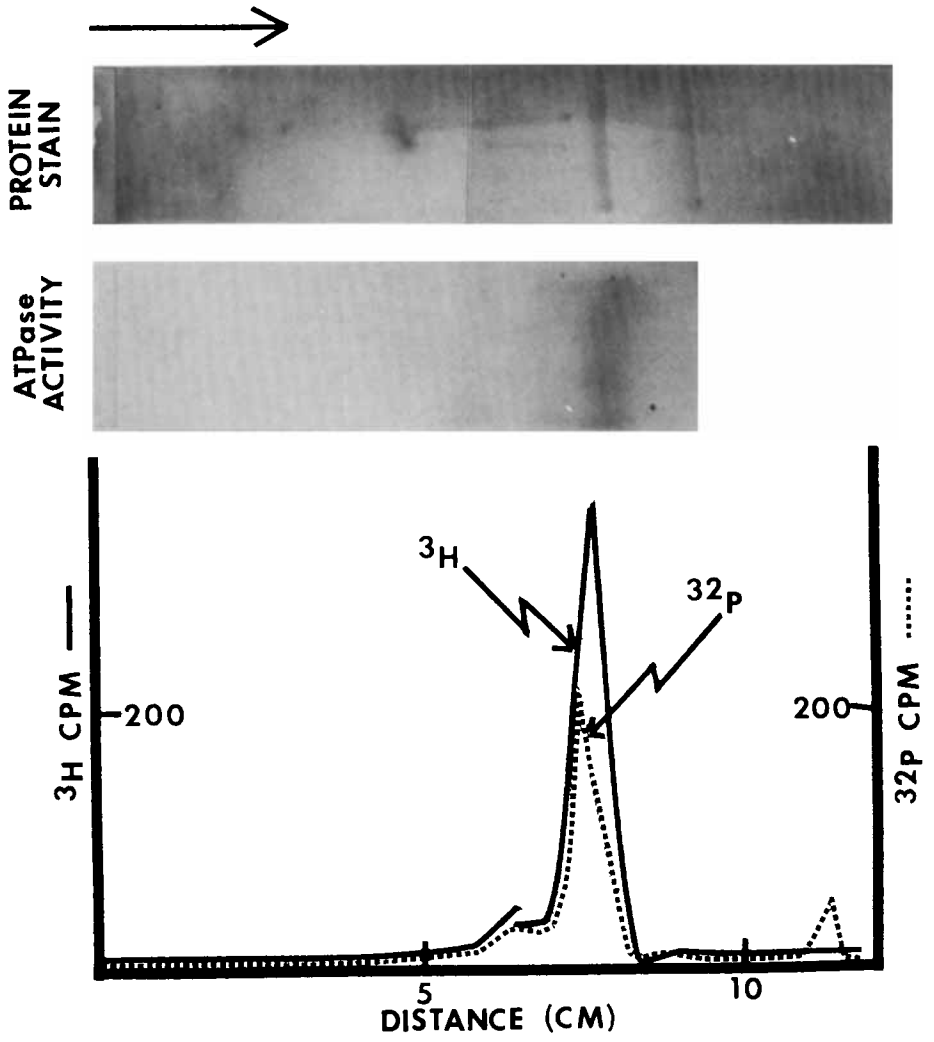

Fig. 8. Polyacrylamide gel analysis of the doubly labeled ATPase-nucelotide complex. Note that the inactive protein has neither endogenous label, ^{32}P, or exogenous label. The analytical procedure for determining the radioactive profile was described previously (16).

ACKNOWLEDGMENTS

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